Termination of translation in eukaryotes is governed by two interacting polypeptide chain release factors, eRF1 and eRF3

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Termination of translation in higher organisms is a GTP-dependent process. However, in the structure of the single polypeptide chain release factor known so far (eRF1) there are no GTP binding motifs. Moreover, in prokaryotes, a GTP binding protein, RF3, stimulates translation termination. From these observations we proposed that a second eRF should exist, conferring GTP dependence for translation termination. Here, we have shown that the newly sequenced GTP binding Sup35-like protein from *Xenopus laevis*, termed eRF3, exhibits *in vitro* three important functional properties: (i) although being inactive as an eRF on its own, it greatly stimulates eRF1 activity in the presence of GTP and low concentrations of stop codons, resembling the properties of prokaryotic RF3; (ii) it binds and probably hydrolyses GTP; and (iii) it binds to eRF1. The structure of the C-domain of the *X.laevis* eRF3 protein is highly conserved with other Sup35-like proteins, as was also shown earlier for the eRF1 protein family. From these and our previous data, we propose that yeast Sup45 and Sup35 proteins belonging to eRF1 and eRF3 protein families respectively are also yeast termination factors. The absence of structural resemblance of eRF1 and eRF3 to prokaryotic RF1/2 and RF3 respectively, may point to the different evolutionary origin of the translation termination machinery in eukaryotes and prokaryotes. It is proposed that a quaternary complex composed of eRF1, eRF3, GTP and a stop codon of the mRNA is involved in termination of polypeptide synthesis in ribosomes.
Keywords: GTP binding protein/protein biosynthesis/termination of translation/vertebrate peptide chain release factor 3 (eRF3)

Introduction

In higher organisms, one polypeptide chain release factor (eRF) governs the hydrolysis of the last peptidyl-tRNA in ribosomes with release of the nascent polypeptide chain in the presence of one of the three termination (stop) codons UAA, UGA, UAG and GTP (reviewed in Caskey, 1980; Craigen *et al.*, 1990). Although functionally the existence of eRF was identified long ago (Goldstein *et al.*, 1970; Konecki *et al.*, 1977), the first attempt (Lee *et al.*, 1990) to reveal its structure failed (Frolova *et al.*, 1993a,b; Timchenko and Caskey, 1994). However, by application of another technique, a family of eukaryotic RFs was identified (eRF1) and the structure of human and frog eRF1 elucidated (Frolova *et al.*, 1994). As anticipated, the eRF1 from *Homo sapiens* and *Xenopus laevis* recognizes all three stop codons in a ribosome- and Mg^{2+}-dependent reaction. It was also proposed (Frolova *et al.*, 1994) that yeast Sup45 protein should be an eRF1, since it belongs to the same structural family. We (Frolova *et al.*, 1994) and others (Stansfield and Tuite, 1994a in comments to our paper quoted above) suggested that a second eRF should exist in addition to eRF1. Two main arguments are in favour of this assumption: in prokaryotes, besides RF1 and RF2, which each recognize two out of the three stop codons (reviewed in Caskey, 1980; Craigen *et al.*, 1990), a third factor, termed RF3, has been identified in *Escherichia coli* that stimulates the termination reaction and binds guanine nucleotides but is not codon-specific (Milman *et al.*, 1969). The *E.coli* gene encoding RF3 has been sequenced and it has been shown that this protein exhibits GTP binding motifs and confers GTP dependence on the termination process (Grentzmann *et al.*, 1994; Mikuni *et al.*, 1994). If termination of translation in prokaryotes and eukaryotes is similar, one may anticipate the existence of a second eRF, since no GTP binding motifs were identified in eRF1 (Frolova *et al.*, 1994), while it has been demonstrated earlier that in mammals termination of translation is a GTP-dependent process (Beaudet and Caskey, 1971; Konecki *et al.*, 1977). Furthermore, we have observed, after the last step of rabbit eRF1 purification (Frolova *et al.*, 1993b, 1994), that its activity decreases significantly and becomes GTP independent.

The aim of this study was to demonstrate the existence of a second eRF that confers GTP dependence on the termination process and to identify its structure. However, it was proposed, based on genetic data, that both yeast Sup45 and Sup35 proteins might be involved in the maintenance of translation fidelity (reviewed in Stansfield and Tuite, 1994b) and/or in translation termination (Inge-Vechtomov and Andrianova, 1970). Since it has been shown earlier (Frolova *et al.*, 1994) that yeast Sup45 protein (Sup45p) belongs to the eRF1 protein family, it seems reasonable to assume that Sup35 protein (Sup35p) could be a candidate for the putative non-identified eRF.

The *Saccharomyces cerevisiae SUP35* gene has been identified and sequenced (Kushnirov *et al.*, 1988). Yeast Sup35p is composed of at least two domains (Kushnirov
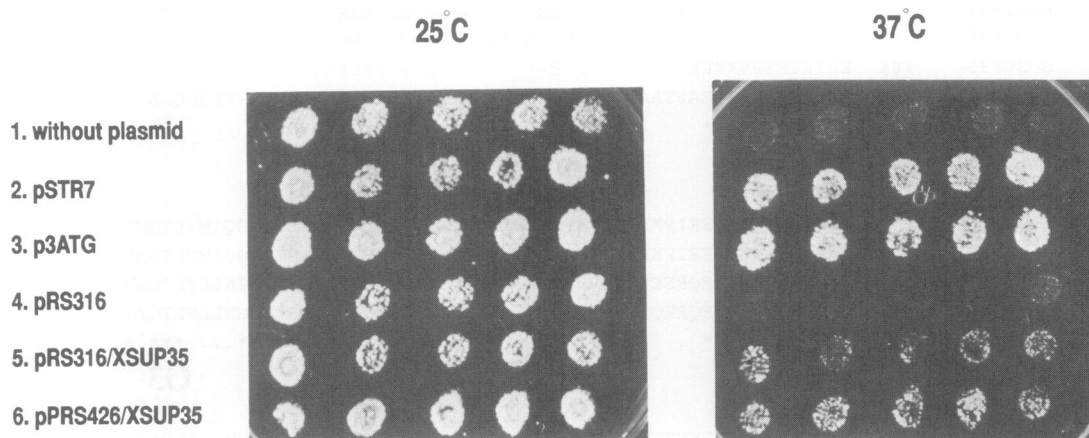
et al., 1988; Kikuchi et al., 1988; Wilson et al., 1988; Ter-Avanesyan et al., 1993): the N- and C-terminal domains (253 and 432 amino acids, respectively). The C-domain exhibits GTP binding motifs (Kushnirov et al., 1988; Kikuchi et al., 1988; Wilson and Cuthbertson, 1988; Samsonova et al., 1991). The C-domain of the Sup35-like proteins is conserved even between distant species like yeast and human (Hoshino et al., 1989) while the N-terminal parts of these proteins are entirely different. The functional dissimilarity of the N- and C-domains of yeast Sup35p has been discussed thoroughly (Doel et al., 1994; Ter-Avanesyan et al., 1994).

Here we demonstrate that the C-domain of X.laevis Sup35-like protein, structurally similar to the yeast Sup35p, is directly implicated in translation termination and confers GTP dependence to the overall termination process.

Results

Isolation and nucleotide sequence analysis of the X.laevis cDNA encoding Sup35-like protein

Using yeast genomic SUP35 DNA (Kushnirov et al., 1988) and GSPT1 cDNA, the human analogue of yeast SUP35 (Hoshino et al., 1989), as probes, a X.laevis cDNA library has been screened. DNA prepared from 30 positive clones has been analysed by restriction endonuclease mapping. Since all of them showed similar restriction maps, the clone containing the longest insert (~2.3 kb) has been subcloned into Bluescript and sequenced. Analysis of the nucleotide sequence (2325 bp) of the cloned cDNA reveals an open reading frame of 1842 nucleotides starting from the 5′ end of the sequence where the initiator Met is absent and ending at the TAA termination codon (nucleotide positions 1843–1845); the AATAAA consensus polyadenylation signal (nucleotide positions 2289–2894) was located 10 nucleotides upstream from the poly(A)$^+$ tail (Figure 1).

Comparison of the primary structure of the C-domains of the SUP35-like proteins

The deduced amino acid sequence of the C-domain of the X.laevis Sup35-like protein (designated as Sup35Cp) is similar to that of human GSPT1p (Hoshino et al., 1989), S.cerevisiae (Kushnirov et al., 1988) and Pichia pinus (Kushnirov et al., 1990) Sup35p (Figure 2), indicating the high conservation of the primary structure of this domain. All these proteins exhibit GTP binding motifs (G1–G4) in the C-domain (Figure 2) similar to those in the other G proteins, in particular in elongation factor EF-1α (Kushnirov et al., 1988; Samsonova et al., 1991) and prokaryotic RF3 (Grentzmann et al., 1994; Mukini et al., 1994), that have to be responsible for guanine nucleotide binding and GTP hydrolysis (reviewed in Bourne et al., 1991). For further experiments, a construct containing the cDNA encoding the X.laevis Sup35Cp starting from Met116 (Figure 1) has been generated and designated as XSUP35C cDNA.

Complementation of yeast sup35(ts) mutation by Xenopus SUP35C

Yeast strain 2-2-33G-D373 containing the sup35 mutation was transformed with multicopy or centromeric plasmids

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ATA ACG GGT ACA ACA CTC TTC CCT CCC ACG TGG GAA GTC CTC CCC ACA CTG CCG ACT CCT   60
 I   T   G   T   T   L   F   P   P   P   T   W   E   V   L   P   T   L   P   T   P    20

TGC CTT ACT CCT TCA GCT CCC CTG ATA AAG CAG TTG GTA TAT CCC AAC CCG ACA CAC CCG  120
 C   L   T   P   S   A   P   L   I   K   Q   L   V   Y   P   N   P   T   H   P    40

GAA ATG GAC GCG AGC GAC TCG GCC CCA GAC TCC TGG GAA CAG GCG GAC ATG GAG GCC ACG  180
 E   M   D   A   S   D   S   A   P   D   S   W   E   Q   A   D   M   E   A   T    60

GAG GCG CAG TTG AAT AAT TCT ATG GCC GCA CTC AAC GTG AAC GCT AAG CCG TTT GTC CCC  240
 E   A   Q   L   N   N   S   M   A   A   L   N   V   N   A   K   P   F   V   P    80

AAT GTT AAC GCC GTA GAG TTC GTG CCG TCC TTC TTG GTG GGC AGC GCG CCA TCG GAC GGG  300
 N   V   N   A   V   E   F   V   P   S   F   L   V   G   S   A   P   S   D   G   100

CAG GAG GAG GAG CAG CAG CAG CAG GGT TCT TGC ACT ACT GTT GGC ATG GAT GTT TCT GAG  360
 Q   E   E   E   Q   Q   Q   Q   G   S   C   T   T   V   G   M   D   V   S   E   120

CCT GTT GTG GAA AAT GGG GAA ACC GAA ATG TCA CCA GAA GAA TCT TGG GAT CAC AAA GAA  420
 P   V   V   E   N   G   E   T   E   M   S   P   E   E   S   W   D   H   K   E   140

GAG CCC ATA GAA GCA GAA CCA GGA GGC GGT TCT GAG GGT GAT GGA GGG ACT ACT GAA GAA  480
 E   P   I   E   A   E   P   G   G   G   S   E   G   D   G   G   T   T   E   E   160

GGA ACC TCT GAA ATG ATG GAG GAA GAG GAG GAA ATT CCA AAA CCC AAA ACA ATT GTT GTA  540
 G   T   S   E   M   M   E   E   E   E   I   P   K   P   K   T   I   V   V     180

CCT CCA GAT GCC CCA AAG AAA GAA CAT GTA AAT GTA GTG TTC ATT GGA CAT GTA GAT GCT  600
 P   P   D   A   P   K   K   E   H   V   N   V   V   F   I   G   H   V   D   A   200

GGT AAA TCA ACC ATT GGT GGG CAA ATA ATG TAT TTA ACA GGC ATG GTT GAA AAA AGA ACT  660
 G   K   S   T   I   G   G   Q   I   M   Y   L   T   G   M   V   E   K   R   T   220

CTT GAA AAA TAT GAA AGA GAA GCC AAG GAA AAA AAT AGA GAA ACC TGG TAT TTG TCA TGG  720
 L   E   K   Y   E   R   E   A   K   E   K   N   R   E   T   W   Y   L   S   W   240

GCT TTA GAC ACA AAT CAG GAA GAA CGA GAC AAA GGC AAA ACT GTA GAG GTT GGT CGG GCC  780
 A   L   D   T   N   Q   E   E   R   D   K   G   K   T   V   E   V   G   R   A   260

TAT TTT GAA ACG GAG AAA AAG CAC TTT ACT ATC CTA GAC GCC CCA GGC CAC AAG AGC TTT  840
 Y   F   E   T   E   K   K   H   F   T   I   L   D   A   P   G   H   K   S   F   280

GTC CCA AAT ATG ATA GGG GGA GCT TCT CAA GCT GAT CTT GCT GTT TTG GTA ATA TCT GCA  900
 V   P   N   M   I   G   G   A   S   Q   A   D   L   A   V   L   V   I   S   A   300

AGG AAA GGA GAA TTC GAA ACT GGC TTT GAA AAA GGA GGA CAG ACA AGA GAA CAT GCC ATG  960
 R   K   G   E   F   E   T   G   F   E   K   G   G   Q   T   R   E   H   A   M   320

TTA GCA AAG ACA GCC GGT GTA AAA CAC TTA ATA GTT CTC ATT AAT AAA ATG GAT GAT CCA 1020
 L   A   K   T   A   G   V   K   H   L   I   V   L   I   N   K   M   D   D   P   340

ACA GTG AAC TGG AGC AAT GAC AGA TAC GAA GAA TGT AAA GAA AAA CTA GTG CCG TTT TTG 1080
 T   V   N   W   S   N   D   R   Y   E   E   C   K   E   K   L   V   P   F   L   360

AAG AAA GTT GGT TTC AAC CCC AAA AAA GAT ATT TAT TTC ATG CCA TGT TCA GGG CTT ACT 1140
 K   K   V   G   F   N   P   K   K   D   I   Y   F   M   P   C   S   G   L   T   380

GGA GCT AAC CTG AAG GAA CCA GTG GAA ACA TGT CCA TGG TAC ATT GGC TTG GCA TTT ATT 1200
 G   A   N   L   K   E   P   V   E   T   C   P   W   Y   I   G   L   A   F   I   400

TCA TAT CTG GAT AGT TTG CCA AAC TTT AAT AGA TCA TTG GAT GGA CCT GTC CGA CTG CCA 1260
 S   Y   L   D   S   L   P   N   F   N   R   S   L   D   G   P   V   R   L   P   420

ATT GTT GAT AAG TAC AAG GAT ATG GGA ACA GTG ATT CTA GGA AAG CTA GAA TCT GGA TCT 1320
 I   V   D   K   Y   K   D   M   G   T   V   I   L   G   K   L   E   S   G   S   440

ATT TGT AAA GGA CAA CAA CTT GCC ATG ATG CCA AAC AAG CAC ATT GTT GAA GTT CTC AGC 1380
 I   C   K   G   Q   Q   L   A   M   M   P   N   K   H   I   V   E   V   L   S   460

CTC CTT TCT GAT GAA GTA GAG ACA GAG CTT GTA GCA CCA GGT GAG AAC CTG AAG CTC CGA 1440
 L   L   S   D   E   V   E   T   E   L   V   A   P   G   E   N   L   K   L   R   480

CTC AAA GGA ATA GAA GAG GAA GAA ATC TTA CCA GGA TTT ATA CTG TGT GAC CCA AAC AAC 1500
 L   K   G   I   E   E   E   E   I   L   P   G   F   I   L   C   D   P   N   N   500

CTT TGT CAT TCT GGA CGT ACA TTT GAT GCC CAG ATT GTG ATA ATT GAG CAC AAA TCT ATC 1560
 L   C   H   S   G   R   T   F   D   A   Q   I   V   I   I   E   H   K   S   I   520

ATC TGC CCT GGT TAC AAT GCA GTG CTG CAT ATT CAT ACC TGT ATT GAA GAA GTC GAA GTA 1620
 I   C   P   G   Y   N   A   V   L   H   I   H   T   C   I   E   E   V   E   I   540

ACG GCC TTA ATC TGT ATG GTA GAC AAA AAA TCA GGA GAG AAA AGT AAG ACG CGA CCC CGA 1680
 T   A   L   I   C   M   V   D   K   K   S   G   E   K   S   K   T   R   P   R   560

TTT GTA AAG CAA GAC CAA GTA TGC ATT GCC CGT CTA AGG ACA GCC GGA ACA ATT TGC CTT 1740
 F   V   K   Q   D   Q   V   C   I   A   R   L   E   T   A   G   T   I   C   L   580

GAA ACA TTC AAA GAT TTC CCT CAG ATG GGA CGA TTT ACC TTA AGA GAT GAA GGT AAG ACC 1800
 E   T   F   K   D   F   P   Q   M   G   R   F   T   L   R   D   E   G   K   T   600

ATT GCA ATT GGA AAG GTC TTG AAA CTG GTT CCA GAA AAG GAC TAA gcttctcaatgctgcacca 1865
 I   A   I   G   K   V   L   K   L   V   P   E   K   D   ***                     614

cagagtgtggaaaactgactctacagaagccgacttcacattgccttcttgttgttctgccccattgacaaaatgattat 1945
ccaagagcagtttgacagcagaaataagtccacattgtcagctttctcatattgagagctctgctatgccactcttcaa 2025
tttccctccctgaacgtttttccaccccttctaaattccgcttcctacgagagaactggcaatagcttcataagtttat 2105
gtggactaaattgcctacaataatgaaaacctaagagattttttttttcttttctttttaaacaagtacattcaatatca 2185
ctcttttttttttcccccctgacctccagaacgagacgacttaattctaggcatattattcaacgaatgtgcgaatgtgtg 2265
agcacgttacaaagacaaactaccgtgttaataaaatattcacttaaaaaaaaaaaaaa                        2325
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Fig. 1. Nucleotide sequence of cDNA encoding the X.laevis Sup35p and the deduced amino acid sequence (GenBank accession number L37045). The initiator methionine used in the pET21b–SUP35 construct is underlined. The termination codon is indicated by asterisks.

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HsGSPT1                         MELSEPI--VENGETEMSPEESWEHKEEISEAEP--GGGSLGDGRPP   43
XlSUP35                         MDVSEPV--VENGETEMSPEESWDHKEEPIEAEP--GGGSEGDGGTT   43
ScSUP35     184  EPTKVEEPVKKEE----KPV-QTE-----EKTEEKS--ELPKVED-LKISESTHNTNNAN   231
PpSUP35     230  AENKVEEESKVEAPTAAKPVSESEFPASTPKTEAKASKEVAAAAAALK-KEVSQAKKESN   289
                 ****  *  *      *        *       *
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G1

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HsGSPT1          EESAHEMMEEEEEIPKPKSVVAPPGAPKKEHVNVVFIGHVDAGKSTIGGQIMYLTGMVDK   103
XlSUP35          EEGTSEMMEEEEEIPKPKTIVVPPDAPKKEHVNVVFIGHVDAGKSTIGGQIMYLTGMVEK   103
ScSUP35          --VTSADALIKEQEEEVDDEVVNDMFGGKDHVSLIFMGHVDAGKSTMGGNLLYLTGSVDK   289
PpSUP35          --VTNADALVKEQEEQIDASIVNDMFGGKDHMSIIFMGHVDAGKSTMGGNLLFLTGAVDK   347
                   . . .   .* .   ..*    .  * *... * ******** **....*** *.*
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G2   ## G3

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HsGSPT1          RTLEKYEREAKEKNRETWYLSWALDTNQEERDKGKTVEVGRAYFETEKKHFTILDAPGHK   163
XlSUP35          RTLEKYEREAKEKNRETWYLSWALDTNQEERDKGKTVEVGRAYFETEKKHFTILDAPGHK   163
ScSUP35          RTIEKYEREAKDAGRQGWYLSWVMDTNKEERNDGKTIEVGKAYFETEKRRYTILDAPGHK   349
PpSUP35          RTVEKYEREAKDAGRQGWYLSWIMDTNKEERNDGKTIEVGKSYFETDKRRYTILDAPGHK   407
                 **.******** * ***** .***.***..***.***..****.*...*********
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G4

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HsGSPT1          SFVPNMIGGASQADLAVLVISARKGEFETGFEKGGQTREHAMLAKTAGVKHLIVLINKMD   223
XlSUP35          SFVPNMIGGASQADLAVLVISARKGEFETGFEKGGQTREHAMLAKTAGVKHLIVLINKMD   223
ScSUP35          MYVSEMIGGASQADVGVLVISARKGEYETGFERGGQTREHALLAKTQGVNKMVVVVNKMD   409
PpSUP35          LYISEMIGGASQADVGVLVISSRKGEYEAGFERGGQSREHAILAKTQGVNKLVVVINKMD   467
                 ....******** .***** **** * *** ***.****.****.**....*..****
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HsGSPT1          DPTVNWSNERYEECKEKLVPFLKKVGFNPKKDIHFMPCSGLTGANLKEQSDF--CPWYIG   281
XlSUP35          DPTVNWSNDRYEECKEKLVPFLKKVGFNPKKDIYFMPCSGLTGANLKEPVET--CPWYIG   281
ScSUP35          DPTVNWSKERYDQCVSNVSNFLRAIGYNIKTDVVFMPVSGYSGANLKDHVDPKECPWYTG   469
PpSUP35          DPTVNWSKERYEECTTKLAMYLKGVGYQ-KGDVLFMPVSGYTGAGLKERVSQKDAPWYNG   526
                 *******..** .*       *  ..  .* *. *** ** .**.**..   *** *
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HsGSPT1          LPFIPYLDNLPNFNRSVDGPIRLPIVDKYKDMGTVVLGKLESGSICKGQQLVMMPNKHNV   341
XlSUP35          LAFISYLDSLPNFNRSLDGPVRLPIVDKYKDMGTVILGKLESGSICKGQQLAMMPNKHIV   341
ScSUP35          PTLLEYLDTMNHVDRHINAPFMLPIAAKMKDLGTIVEGKIESGHIKKGQSTLLMPNKTAV   529
PpSUP35          PSLLEYLDSMPLAVRKINDPFMLPISSKMKDLGTVIEGKIESGHVKKGQNLLVMPNKTQV   586
                 ... ***..  *  . * .*** .* **.**.. **.*** . *** .**** *
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HsGSPT1          EVLGILSD-DVETDTVAPGENLKIRLKGIEEEEILPGFILCDPNNLCHSGRTFDAQIVII   400
XlSUP35          EVLSLLSD-EVETELVAPGENLK-RLKGIEEEEILPGFILCDPNNLCHSGRTFDAQIVII   400
ScSUP35          EIQNIYNETENEVDMAMCGEQVKLRIKGVEEEDISPGFVLTSPKNPIKSVTKFVAQIAIV   589
PpSUP35          EVTTIYNETEAEADSAFCGEQVRLRLRGIEEEDLSAGYVLSSINHPVKTVTRFEAQIAIV   646
                 *.  .. .. . *. * ** .*..*.***.. .*..* ... .. * ***.*.
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HsGSPT1          EHKSIICPGYNAVLHIHTCIEEVEITALICLVDKKSGEKSKTRPRFVKQDQVCIARLRTA   460
XlSUP35          EHKSIICPGYNAVLHIHTCIEEVEITALICMVDKKSGEKSKTRPRFVKQDQVCIARLETA   460
ScSUP35          ELKSIIAAGFSCVMHVHTAIEEVHIVKLLHKLEKGTNRKSKKPPAFAKKGMKVIAVLETE   649
PpSUP35          ELKSILSTGFSCVMHVHTAIEEVTFTQLLHNLQKGTNRRSKKAPAFAKQGMKIIAVLETT   706
                 * ***. .*.. * *.** ****  .. *.  ..*..  .**. * *.*..  ** * *.
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HsGSPT1          GTICLETFKDFPQMGRFTLRDEGKTIAIGKVLKLVPEKD                       499
XlSUP35          GTICLETFKDFPQMGRFTLRDEGKTIAIGKVLKLVPEKD                       499
ScSUP35          APVCVETYQDYPQLGRFTLRDQGTTIAIGKIVKIA-E                         685
PpSUP35          EPVCIESYDDYPQLGRFTLRDQGQTIAIGKVTKLL                           741
                 ...*.*...*.** ******* * ******. *.
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Fig. 2. Alignment of the predicted amino acid sequences of human HsGSPT1 (Hoshino *et al.*, 1989), *X.laevis* XlSUP35 (this work), *S.cerevisiae* ScSUP35 (Kushnirov *et al.*, 1988) and *P.pinus* PpSUP35 (Kushnirov *et al.*, 1990) Sup35Cp. Conserved G domain motifs G1–G4 (Bourne *et al.*, 1991) are indicated by solid lines, identical amino acids by asteriks and similar amino acids by dots.

carrying yeast *SUP35* or *XSUP35C*. Leu[+] (for pSTR7) or Ura[+] (for other plasmids) transformants were selected at 25°C and then tested at the restrictive temperature (37°C).

The results of this experiment are presented in Figure 3. Analysis of the transformants obtained showed that plasmids carrying *XSUP35C* under the control of the yeast

Fig. 3. Complementation of the yeast *sup35(ts)* mutation by *X.laevis SUP35C*. The *S.cerevisiae* temperature-sensitive strain 2-2-33G-D373 was transformed by plasmids indicated in the key. Five Leu$^+$ or Ura$^+$ transformants in each case were replica plated on YPD plates and incubated at 25°C or 37°C.

SUP35 promoter are able to compensate temperature sensitivity caused by the *sup35* mutation in *S.cerevisiae*, although less efficiently than plasmids with the yeast *SUP35* gene. Better compensation was obtained with the multicopy plasmid pRS426/XSUP35 carrying the promoter of yeast *SUP35* than with the centromeric plasmid pRS316/XSUP35 with the same promoter. Thus, genetic complementation experiments indicate the functional similarity between yeast *SUP35* and *X.laevis SUP35C*, as was previously shown for the human *GSPT1* gene (Hoshino *et al.*, 1989).

RF-stimulating acivity of X.laevis Sup35Cp and complex formation between X.laevis Cl1 (eRF1) and Sup35C proteins

The *E.coli*-expressed and affinity-purified *X.laevis* Sup35Cp yields one predominant band (~58 kDa) on SDS–PAGE after Coomassie blue staining (Figure 4, lane 1). The purified Sup35Cp tested in the *in vitro* RF assay (Table I) possesses no RF activity but it greatly enhances the activity of *X.laevis* Cl1 protein (eRF1) at non-saturating levels of all three stop codons. This stimulation is entirely GTP dependent and is inhibited by the non-hydrolysable analogue of GTP, GTPγS (Table II), while GDP, GMP or ATP have no effect (Table III). The Cl1 (eRF1) activity is not affected by GTP and this protein remains active in the presence of GTPγS (Table II). Rabbit polyclonal antibodies raised against *X.laevis* Sup35Cp significantly inhibit the RF-stimulating activity of Sup35Cp and do not diminish the activity of Cl1 (eRF1) (Table IV).

The strong stimulation of Cl1 (eRF1) activity in the presence of Sup35Cp might be caused by mutual interaction of these proteins. As shown in Figure 5, the addition of *E.coli*-expressed His-tagged Cl1 (eRF1) protein to *Xenopus* egg extract leads to complex formation between exogeneous Cl1 protein and endogeneous *Xenopus* Sup35p present in this extract. Sup35p has been detected by immunoblot assay in the bound form with Cl1 protein after immobilization of His-tagged Cl1 protein onto a NiNTA column and its subsequent elution. Thus, *X.laevis* His-tagged Cl1 protein and Sup35p bind to each other.

The immunoblot analysis of *X.laevis* egg extracts with polyclonal antibodies against *X.laevis* Sup35Cp demon-
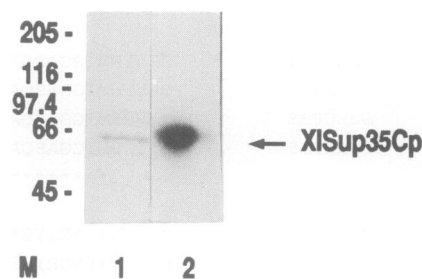
Fig. 4. SDS–PAGE (8% acrylamide) analysis of purified *X.laevis* Histagged Sup35Cp. M, molecular weight markers (K); lane 1, Sup35Cp after Coomassie blue staining; lane 2, Sup35Cp revealed by Western blot.

strates that the protein product of the *SUP35* gene is much longer than Sup35Cp (80 kDa versus 58 kDa) (Figure 5, lane 3); the same result was obtained with rabbit cell extracts (not shown). Consequently, the size of yeast, rabbit and *X.laevis* Sup35p are similar, indicating the two-domain organization of Sup35p.

Discussion

In mammals, the release of a polypeptide chain from peptidyl-tRNA at the last step of protein synthesis is a GTP-dependent process (Beaudet and Caskey, 1971; Konecki *et al.*, 1977). In all known cases when GTP is required for some biochemical reactions to proceed, GTP binding motifs have been identified (for review, see Bourne *et al.*, 1991). However, the eRF1 family does not exhibit any GTP binding motifs (Frolova *et al.*, 1994). This apparent discrepancy can be resolved easily if one assumes the involvement of an additional eRF capable of binding GTP that confers GTP dependence on the overall termination process. We have demonstrated here (Table II) that in fact eRF1-controlled termination is entirely GTP independent, as anticipated from the lack of GTP binding motifs in the eRF1 amino acid sequence. This holds true for all three stop codons. However, in the presence of another vertebrate protein, *Xenopus* Sup35Cp, structurally similar to the C-domain of yeast Sup35p, the termination reaction is greatly stimulated by GTP, while its non-

Table I. Effects of *X.laevis* Sup35Cp on the *in vitro* RF activity of *X.laevis* Cl1 (eRF1) protein

Protein	Sup35Cp added (μg)	f[³⁵S]Met released, pmol					
		Stop codon added (μM)					
		UAAA		UAGA		UGAA	
		50	5	50	5	50	5
Cl1	–	0.44	0.01	0.71	0.02	0.48	0.01
–	0.3	0	0	0	0	0	0
Cl1	0.1	–	0.38	–	0.50	–	0.37
Cl1	0.2	–	0.51	–	0.85	–	0.51
Cl1	0.3	–	0.56	–	0.88	-	0.63

Table II. Influence of GTP and GTPγS on the RF activity of Cl1 (eRF1) protein and the RF-stimulating activity of Sup35Cp

Protein(s)	Guanine nucleotide added (0.1 mM)	f[³⁵S]Met released, pmol		
		Stop codon added		
		UAAA	UGAA	UAGA
Cl1	None	1.00	0.87	0.90
Cl1	GTP	0.86	1.01	0.75
Cl1	GTPγS	0.82	0.93	0.72
Cl1 + Sup35Cp	None	0.02	0.05	0.01
Cl1 + Sup35Cp	GTP	0.97	0.93	0.88
Cl1 + Sup35Cp	GTPγS	0.05	0.06	0.03

The RF activity of Cl1 protein (eRF1) and RF-stimulating activity of Sup35Cp (0.3 μg) towards Cl1 protein were measured at saturation and non-saturation levels of all three termination codons respectively.

Table III. Comparison of the effects of nucleotides on RF-stimulating activity of *X.laevis* Sup35Cp

Protein	Sup35Cp added (0.2 μg)	Stop codon added (5 μM)	f[³⁵S]Met released, pmol			
			Nucleotide added			
			GTP	GDP	GMP	ATP
Cl1	–	UAAA	0.08	0.09	0.02	0.05
Cl1	+	UAAA	0.70	0.04	0.04	0.05
Cl1	–	UAGA	0.01	0.01	0.03	0.01
Cl1	+	UAGA	0.40	0.03	0.04	0.03
Cl1	–	UGAA	0.01	0.01	0.03	0.01
Cl1	+	UGAA	0.61	0.03	0.04	0.03

Table IV. Effects of anti-Sup35Cp antibodies on the RF activity of Cl1 (eRF1) protein and RF-stimulating activity of Sup35Cp

Proteins	UAAA (μM)	Anti-Sup35Cp antibodies added (μl)	f[³⁵S]Met released, pmol
Cl1	50	–	0.40
Cl1	50	2	0.37
Cl1	50	4	0.34
Cl1	50	6	0.40
Cl1 + Sup35Cp	5	–	0.65
Cl1 + Sup35Cp	5	2	0.68
Cl1 + Sup35Cp	5	4	0.22
Cl1 + Sup35Cp	5	6	0.10

The amounts of Cl1 (eRF1) and Sup35Cp proteins used in this experiment were 0.2 μg and 0.3 μg respectively.
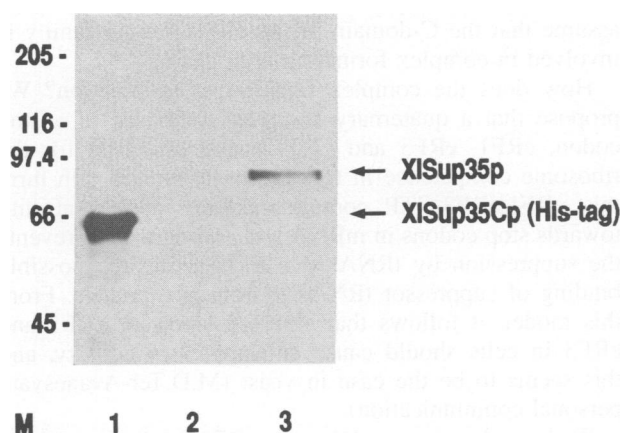
Fig. 5. SDS–PAGE (8% acrylamide) and Western blot analysis of complex formation between *X.laevis* His-tagged Cl1 protein and Sup35p present in *Xenopus* egg extract. M, molecular weight markers (K); lane 1, the purified His-tagged Sup35Cp; lane 2, the purified His-tagged Cl1 protein kept with buffer and passed through the column; eluted fraction (control); lane 3, the same as in lane 2 but after incubation of the purified His-tagged Cl1 protein with *Xenopus* egg extract.

hydrolysable analogue, GTPγS, is entirely inactive (Table II). From this observation, it is clear that the GTP requirement of the overall termination reaction is conferred by another protein possessing GTP binding motifs.

Since, in the presence of non-hydrolysable analogue of GTP, GTPγS, *X.laevis* Sup35Cp is inactive, we assume that GTP is hydrolysed in the course of the overall termination reaction. Remarkably, the C-domain of *Xenopus* Sup35p alone is entirely inactive in promoting peptidyl-tRNA hydrolysis. From these data, one may anticipate that eRF1 binds to another protein, termed here as eRF3 for the reasons explained below.

Besides conferring GTP dependence, the second protein, eRF3, allows the concentration of the stop codons in the *in vitro* RF assay governed by eRF1 to be reduced considerably (Table I). Consequently, two features of *X.laevis* Sup35Cp/eRF3 (GTP binding motifs and stimulation of RF activity at limiting concentrations of stop

codons) resemble those of *E.coli* RF3 (Grentzmann *et al.*, 1994; Mukini *et al.*, 1994). For these reasons, we term *X.laevis* GTP binding protein and other proteins of this family (Figure 2) as eRF3. However, eRF3 and prokaryotic RF3 exhibit certain distinct properties: GTP enhances the eRF3 activity but inhibits the prokaryotic RF3 activity (Grentzmann *et al.*, 1994; Mukini *et al.*, 1994) and part of the yeast *SUP35*-like gene encoding Sup35Cp is essential for cell viability (Kushnirov *et al.*, 1988) while the *E.coli* gene encoding RF3 is non-essential (Grentzmann *et al.*, 1994; Mukini *et al.*, 1994). Obviously, since yeast Sup35p belongs to the same structural family (Figure 2) it should also be termed as eRF3/Sup35p, although it has not yet been demonstrated in the appropriate assay system. Based on our previous results (Frolova *et al.*, 1994), the same suggestion concerning Sup35p has been made by Stansfield and Tuite (1994a) but not proven biochemically.

In this paper, we do not consider the role of the N-domain in the function of Sup35p. It is known that this part of yeast Sup35p might be functionally antagonistic towards its own C-domain (Doel *et al.*, 1994; Ter-Avanesyan *et al.*, 1994). Moreover, the properties of the full-length Sup35p of higher eukaryotes might differ from

the functional activity of the C-domain; these aspects of the function of Sup35p in higher eukaryotes will be considered elsewhere.

We conclude that, in vertebrates (and most probably in eukaryotes in general), two factors (eRF1 and eRF3) govern the translation termination process as in prokaryotes (RF1/RF2 and RF3). The first factor, eRF1, recognizes directly or indirectly all three stop codons in mRNA and catalyses the peptidyl-tRNA hydrolysis, while this reaction proceeds more efficiently and becomes GTP dependent due to participation of the second factor, eRF3, in the overall reaction. When two proteins are involved in one and the same reaction, one may anticipate their interaction. In fact, we observed (Figure 5) that *X.laevis* eRF1 and eRF3 bind to each other. This observation provokes our assumption that eRF1 and eRF3 are also able to form a complex *in vivo*. To be more precise, we assume that the C-domain of the eRF3 protein family is involved in complex formation with eRF1.

How does the complex function in termination? We propose that a quaternary complex composed of a stop codon, eRF1, eRF3 and GTP is a prerequisite for the ribosome competence in translation termination. In turn, this eRF1/eRF3/GTP complex exhibits higher affinity towards stop codons in mRNA and consequently prevents the suppression by tRNAs due to blocking the possible binding of suppressor tRNAs to nonsense codons. From this model, it follows that elevated levels of eRF1 and eRF3 in cells should cause antisuppressor activity, and this seems to be the case in yeast (M.D.Ter-Avanesyan, personal communication).

We have demonstrated that the eRF1 family is structurally highly conserved (Frolova *et al.*, 1994). As is shown here (Figure 5), eRF1 binds to the C-domain of eRF3, and therefore one may anticipate the high conservation of eRF3 structure too, at least in the C-domain. This prediction is in agreement with sequence data (Figure 2) and genetic complementation data (Figure 3).

Surprisingly, the primary structure of the eRF1 family is dissimilar to prokaryotic RF1/2 and yeast mitochondrial RF-1 (Frolova *et al.*, 1994). Again, since eRF3 and eRF1 bind to each other, one may expect that eRF3 (C-domain) might be different from prokaryotic RF3. The comparison of the data presented in Figure 2 with the *E.coli* RF3 amino acid sequence (Grentzmann *et al.*, 1994; Mukini *et al.*, 1994) shows that the similarity between eRF3 and *E.coli* RF3 extends only to GTP binding motifs but not to the rest of the molecule (not shown).

The absence of structural resemblance between prokaryotic and eukaryotic RFs, coupled with their functional similarity, raises an intriguing question about the origin of the translation termination machinery in eukaryotes. One of the ideas proposed earlier (Frolova *et al.*, 1994) concerns the origin of prokaryotic and eukaryotic translation systems as being independent processes in evolution.

Materials and methods

Materials

Escherichia coli tRNA^{fMet} and GTPγS were purchased from Sigma, peroxidase-linked swine anti-rabbit IgG from DAKO-Immunoglobulin, ECL Western blot reagents, Hybond C and L-[^{35}S]methionine (37 TBq/mmol) from Amersham, Centricon 10 from Amicon, NiNTA resin from QIAGEN, pET21b plasmid from Novagen, plasmids pRS316 and pRS426 from ATCC (American Type Culture Collection), nitrocellulose filters BA85 from Schleicher & Schuell, and deoxyribo-oligonucleotide primers for the PCR reaction from Bioprobe. Tetraribonucleotides and AUG were synthesized by A.Veniaminova and N.Ryabkova (Novosibirsk State University, Russia).

Yeast strain and media

The *S.cerevisiae* strain 2-2-33G-D373 [*MATα pheA10 ade2-144,717 his7-1 lys9-A21 ura3-52 leu2-3,112 trp1-289 sup35-21(ts)*, Petergoff Genetic Collection of Yeast Strains, St Petersburg University, St Petersburg, Russia] was used for complementation experiments. Standard yeast media were as described by Sherman *et al.* (1986).

Plasmids

Plasmids pSTR7 containing yeast *SUP35* and p3ATG containing yeast *SUP35* starting from the third ATG (*SUP35C*) and encoding the C-domain of the yeast Sup35p were described previously (Telkov *et al.*, 1986; Ter-Avanesyan *et al.*, 1993). A plasmid pCYM1-11 containing *Xenopus SUP35C* (pCYM1-11/XSUP35) was constructed by insertion of a *Hind*II–*Sac*I fragment of Bluescript/XSUP35C into the *Sma*I–*Sac*I sites of pCYM1-11 (Camonis *et al.*, 1990). A plasmid pRS426 containing *Xenopus SUP35C* (pRS426/XSUP35) was constructed by insertion of a 1.8 kb *Bam*HI–*Sac*I fragment of pCYM1-11/XSUP35 into the *Bam*HI–*Sac*I sites of pRS426 (Christianson *et al.*, 1992). The resulting plasmid was digested with *Eco*RI and *Bam*HI and ligated with 0.7 kb *Bam*HI–*Eco*RI PCR product corresponding to yeast *SUP35* promoter (forward primer *Eco*RI, 5'-CGGGAATTCCTTACAACAACGGTC-3' and reverse primer *Bam*HI, 5'-CGCGAATTCCTAGTGGGCAG-3'). A plasmid pRS316/XSUP35 was constructed by insertion of a 2.4 kb *Hind*III fragment of the yeast *SUP35* promoter/XSUP35C cassette from pRS426/pXSUP35 into the *Hind*III site of pRS316 (Sikorski and Hieter, 1989).

Yeast transformation and complementation of yeast *sup35(ts)* mutation by *Xenopus SUP35C*

Yeast transformation was performed according to Ito *et al.* (1983). The *S.cerevisiae* strain 2-2-33G-D373 containing the *sup35(ts)* mutation was transformed with multicopy or centromeric plasmids containing yeast *SUP35* or *Xenopus SUP35C*. Leu$^+$ (for pSTR7) or Ura$^+$ (for other plasmids) transformants were selected at 25°C and then tested at the restrictive temperature (37°C).

Selection and sequencing of *X.laevis SUP35* cDNA

A 1.4 kb *Kpn*I–*Kpn*I fragment of *S.cerevisiae SUP35* DNA (Kushnirov *et al.*, 1988) and a 1.8 kb *Bam*HI–*Dra*I fragment of human *GSPT1* cDNA (Hoshino *et al.*, 1989) were used as hybridization probes for screening a *X.laevis* egg cDNA library in λgt10 (Paris and Philippe, 1990). About 1.5×10^5 plaques were screened by hybridization of nitrocellulose filters (BA85) at 55°C in 6× SSC, 5× Denhardt's, 0.5% SDS to yield 30 positive clones. The selected positive clone with the longest cDNA was recloned into Bluescript and sequenced on both strands by the dideoxy chain termination method (Sanger *et al.*, 1977).

The expression and purification of the *X.laevis* CI1 (eRF1) and Sup35C proteins

The expression and purification of *X.laevis* CI1 (eRF1) protein were performed as described (Frolova *et al.*, 1994). For the expression of the part of the *X.laevis SUP35* cDNA corresponding to the C-domain of SUP35p starting from Met116 (Figure 1), *X.laevis SUP35* cDNA inserted into Bluescript vector was amplified by PCR using the following oligos: forward primer *Nhe*I, 5'-CCCGCTAGCGATGTTTCTG-3' and reverse primer *Xho*I, 5'-CTTCTCGAGGTCCTTTTCTGG-3'. The amplified product was digested with *Nhe*I and *Xho*I and inserted into *Nhe*I–*Xho*I sites of the expression vector pET21b to give rise to pET21b-SUP35C plasmid. A 6 His-tag tail was present at the C-terminus of the expressed Sup35Cp. *E.coli*, strain BL21(DE3), was transformed with pET21b-SUP35C plasmid and induced by isopropyl-β-D-thiogalactopyranoside (IPTG) to express His-tagged Sup35Cp. NiNTA resin was equilibrated with 5 mM imidazole, and the 8000 *g* supernatant of the cell lysate with expressed *X.laevis* Sup35Cp was passed through the column. The column was washed extensively with 20 mM imidazole and His-tagged Sup35Cp was eluted from the resin with 0.25 M imidazole. Fractions containing Sup35Cp were combined, dialysed against buffer containing 50 mM Tris–HCl pH 7.5, 0.1 M KCl and 2 mM DTT, concentrated on Centricon 10 and stored at –80°C.

Antibodies

The rabbit polyclonal anti-Sup35Cp antibodies were obtained and affinity-purified by the same procedure as for the rabbit polyclonal anti-Cl1 antibodies (Tassan *et al.*, 1993). The purified *X.laevis* Cl1 protein (0.2 µg) or Sup35Cp (0.3 µg) was incubated with increasing amounts of the anti-Sup35Cp antibodies at 4°C for 30 min and then added to the incubation mixture.

Protein gel electrophoresis and Western blot analysis

SDS–PAGE and Western blots were performed according to Laemmli (1970) and Towbin *et al.* (1979) respectively. Proteins were separated on SDS–PAGE, then transferred to nitrocellulose membrane (Hybond C) and a Western blot analysis was performed with 1/1000 diluted affinity-purified anti-Sup35Cp antibodies, and an ECL kit was used for revelation according to the manufacturer's instructions.

Complex formation between X.laevis Sup35 and His-tagged Cl1 (eRF1) proteins

Escherichia coli-expressed and purified His-tagged Cl1 protein (40 µg) was incubated with 0.3 ml of a *Xenopus* egg extract or with the same volume of buffer (50 mM Tris–HCl pH 7.5, 50 mM KCl, 2 mM DTT) in the presence of 1 mM GTP at 22°C for 1 h. Both samples were diluted and loaded separately onto a NiNTA column. After extensive washing of the column with 20 mM imidazole, the His-tagged Cl1 protein was eluted from the column with 1.5 ml of 0.25 M imidazole and concentrated on Centricon 10. Eluted proteins after SDS–PAGE were transferred onto nitrocellulose membranes (Hybond C) and Western blot analysis was performed.

In vitro termination assay

Formyl[^{35}S]methionyl-tRNA^{fMet} was synthesized using *E.coli* tRNA^{fMet} and L-[^{35}S]methionine as described by Tate and Caskey (1990). Rabbit reticulocyte ribosomes were isolated as described by Cox and Hirst (1976) and were kindly provided by E.Davydova (Institute of Protein Research, Pustchino, Russia). The RF-stimulating activity of *X.laevis* Sup35Cp was measured *in vitro* as stop codon-dependent hydrolysis of f[^{35}S]Met-tRNA^{fMet} associated with the AUG–80S ribosome complex (Caskey *et al.*, 1974; Tate and Caskey, 1990). The incubation mixture (25 µl) contained 20 mM Tris–HCl, pH 7.5, 15 mM MgCl$_2$, 8 mM NH$_4$Cl, 0.1 mM GTP, 1.5 pmol f[^{35}S]Met-tRNA^{fMet}–AUG–ribosome complex, 0.2 µg of the *X.laevis* Cl1 protein (eRF1), 5 µM of the stop codon (that corresponded to ~10% of the saturation level needed for the complete fMet release with Cl1 but without Sup35Cp) and indicated amounts of the purified *X.laevis* Sup35Cp. The RF activity of *X.laevis* Cl1 (eRF1) alone (0.2 µg) was measured in the same incubation mixture except that the concentration of the stop codon was 50 µM (saturation level). The RF activity was calculated as the amount of f[^{35}S]Met released in the presence of stop codon; the value (~ 0.05–0.15 pmol) of f[^{35}S]Met released in the absence of stop codon has been subtracted from all values.

Acknowledgements

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